A dimerization model for the concentration dependent photophysical properties of diphenylhexatriene and its phospholipid derivatives

DPHpPC and DPHpPA

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ABSTRACT We have investigated the reason for the sensitivity of the fluorescence excited-state lifetime of 1,6-diphenyl-1,3,5-hexatriene (DPH) and its phospholipid derivatives, 1-palmitoyl-2-({2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]ethyl}carbonyl)-3-*sn*-phosphatidylcholine (DPHpPC) and 1-palmitoyl-2-({2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]ethyl}carbonyl)-3-*sn*-phosphatidic acid (DPHpPA), to the concentration of these probes in dipalmitoylphosphatidylcholine (DPPC) multilamellar membranes (Barrow, D. A., and B. R. Lentz, 1985. *Biophys. J.* 48:221–234; Parente, R. A., and B. R. Lentz. 1985. *Biochemistry.* 24:6178–6185). We have interpreted self-quenching data, excitation and emission spectra, and phase and modulation lifetime data in terms of a model that envisions dimerization of these probes in a membrane bilayer. It is proposed that dimerization alters the symmetry of the DPH excited state so as to allow more rapid decay via the normally symmetry-disallowed route from the $^1Ag^*$ state. Global analysis of fluorescence phase shift and modulation ratio data for DPHpPC in terms of the dimerization model provided a good fit of these data as a function of both modulation frequency and probe concentration. Global analysis of a similar set of data for the charged phosphatide DPHpPA predicted that this probe was much less prone to dimerize than was the uncharged DPHpPC. This physically reasonable result provides support for the assumptions made in the development of our model. We conclude that the dimerization model allows rationalization of many of the anomalous photophysical properties of DPH and its derivatives in membranes.

INTRODUCTION

Diphenylhexatriene (DPH)[1] and its derivatives have been widely used for detecting molecular order and dynamics within the hydrocarbon region of a membrane bilayer (for reviews, see Shinitzky and Barenholz, 1978; Lentz, 1988). However, the use of DPH has also been criticized for a number of reasons, including its uncertain location within the bilayer (Lentz et al., 1976; Davenport et al., 1985) and its anomalous excited-state characteristics. We and others have introduced derivatives of DPH that fix the chromaphore at specific locations within the bilayer (Prendergast et al., 1981; Morgan et al., 1982; Parente and Lentz, 1985), thus answering the first criticism. However, DPH's anomalously long excited-state lifetime (Cundall et al., 1979), its tendency to display complex fluorescence decay characteristics (Chen et al., 1977), and its photosensitivity (Duportail et al., 1983) all make it difficult to obtain detailed interpretations of observed fluorescence properties in terms of molecular motions and organizations in the bilayer.

Recently, we observed that the fluorescence excited state lifetime of DPH (Barrow and Lentz, 1985) and of a phospholipid containing the DPH moiety in the No. 2 acyl chain, DPHpPC, (Parente and Lentz, 1986) showed dramatic quenching at high concentrations in model membranes. We used this observation to develop an empirical assay for the mixing of membrane lipids during membrane fusion (Parente and Lentz, 1986). Our efforts to understand the physical basis of this apparent lifetime quenching led to the model presented here, namely that DPH dimerizes in a membrane to yield a species whose altered excited-state structure accounts for the existence of a low-lifetime species. This model allows us to rationalize many of the anomalous photophysical properties of DPH. Most significantly, this model provides a potential starting point for a quantitative interpretation of observed fluorescence properies of DPH in membranes.

[1] *Abbreviations used in this paper:*
DPH, 1,6-diphenyl-1,3,5-hexatriene; DPHpPA, 1-palmitoyl-2-[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]ethyl]carbonyl]-3-*sn*-phosphatidic acid; DPHpPC, 1-palmitoyl-2-[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl) phenyl]ethyl]carbonyl]-3-*sn*-phosphatidylcholine; DPPC, 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine; LMV, large, multilamellar vesicles; TLC, thin-layer chromatography.

MATERIALS AND METHODS

Ultrapure KCl was from Heico, Inc. (Delaware Water Gap, PA; lot 2179). KCl solutions were twice filtered through a 0.22 μm filter

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(Millipore/Continental Water Systems, Bedford, MA) and degassed before use. Amberized glassware was purchased from Reliance Glass Works, Inc. (Bensenville, IL). All other chemicals were reagent grade or better.

Lipids

A chloroform solution of 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine (DPPC) was purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). DPPC was filtered once before use over Norit-A neutral activated charcoal to remove fluorescent contaminants. DPPC was found to be >98% pure when chromatographed on Analtech (Newark, DE) silica gel thin-layer chromatography (TLC) plates (type GHL, 5 × 20 cm) developed in $CHCl_3$-CH_2OH-H_2O (65:25:4 vol/vol/vol) and stained with iodine. A small amount of 1(4',-(2-carboxyethyl))-DPH was obtained from Dr. E. Thomas (Salford, UK) and converted to DPHpPC and DPHpPA by Dr. Walter Shaw of Avanti Polar Lipids, Inc. TLC plates were run in the solvent system above and visualized by ultraviolet light to reveal fluorescence material followed by iodine staining for lipid. In both cases, only one spot was observed.

Vesicle preparation

Lipid and probe stocks were mixed in various molar ratios and dried under argon onto the wall of a round-bottom flask. The lipid film was redissolved in a small amount of benzene and lyophilized under high vacuum (0.5 mmHg) for 8–12 h to yield a white powder. Large, multilamellar vesicles (LMV) were prepared in 50 mM KCl as described previously (Lentz et al, 1976). Samples were stored under argon and swirled at 50°C in a rotary incubator for at least 2 h before use. DPHpPC was found to be fairly sensitive to photolysis. Thus, samples were prepared and stored in amberized glassware or in the dark to prevent light-induced photolysis. No photolysis was apparent when samples were treated in this manner.

Fluorescence measurements

Unless otherwise stated, all measurements were obtained at 45°C, i.e., above the order/disorder transition temperature of DPPC (41.2°C).

Fluorescence emission spectra were determined on a model 4800 spectrofluorometer (SLM Instruments, Urbana, IL). Fluorescence excitation spectra were recorded on an SLM 48000 spectrofluorometer. Excitation for emission spectra was with the 366 nm mercury line of a 200-W mercury-xenon arc lamp (Canrad-Hanovia, Inc., Newark, NJ). Excitation spectra were recorded using a 150-W high-pressure xenon lamp mounted horizontally in a focused lamp housing (Photon Technology International, Princeton, NJ).

Fluorescence lifetime measurements were performed on the SLM 48000 multifrequency phase and modulation fluorometer. Adequate sample illumination was essential for obtaining accurate lifetime measurements on this instrument. This was accomplished in two ways. First, the light source was a 200-W mercury-xenon arc lamp mounted horizontally in the Photon Technology International focused lamp housing. With this source, we obtained at 366 nm approximately two to three times the effective light throughput of the 450-W mercury-xenon arc lamp supplied with the SLM instrument. Second, a Soliel-Babinet compensator (Karl Lambrecht, Chicago, IL) was used to rotate the exciting beam from the electrooptic modulator to 35° off vertical. This allowed the use of "magic angle" excitation without the excessive loss of light associated with an excitation polarizer. For the present study, a set of 7–16 modulation frequencies was used in the range from 10 to 150 MHz. For each frequency, the phase shift and the modulation ratio of the fluorescence were measured with respect to DPH in heptane as a reference fluorophore of known lifetime (τ = 6.77 ns; 2×10^{-7} M; Barrow and Lentz, 1983). Data were collected on an AT clone computer (Dell Computer Corp., Austin, TX) using software supplied by SLM Instruments and analyzed according to single or double lifetime models as previously described (Gratton et al., 1984). Global analysis of several frequency-resolved data sets as a function of DPHpPC or DPHpPA concentration were performed with a software package obtained from the Laboratory for Fluorescence Dynamics, University of Illinois at Urbana-Champagne. This package is described in detail elsewhere (Beechem and Gratton, 1988).

RESULTS

Fluorescence intensity measurements

Fluorescence intensity was measured for samples containing various molar ratios of DPHpPC. The measured values were plotted according to the Stern-Volmer relation for quenching (Lakowicz, 1983), taking the mole fraction of probe as the quencher concentration. This yielded a plot which was concave up (Fig. 1), indicating complex formation in the ground state. A similar Stern-Volmer-type plot of the phase lifetime at 30 MHz[2] yielded a linear response (Fig. 1), indicating that the observed decrease in fluorescence lifetime with increasing probe concentration approximately followed an excited-state, dynamic quenching model. Clearly, the self-quenching of DPHpPC is a complex process involving a probe–probe complex formation that limits absorption of light as well as some mechanism for enhancing decay of the excited state.

Spectral measurements

Emission spectra were recorded at three probe concentrations (4.0, 0.67, 0.2 mol%; Fig. 2). We observed no shift in the spectra as a function of probe concentration in the membrane. This is not inconsistent with an excited-state reaction mechanism, but if an excited state reaction were responsible for the observed lifetime quenching, the data in Fig. 2 imply that the product of this reaction must have a fluorescence spectrum very similar to that of unreacted DPHpPC. In addition, the fluorescence anisotropy spectra at two probe concentrations (4.0 and 0.25 mol%) are plotted in Fig. 2. Again, the invariance of fluorescence anisotropy with probe concentration (compare open circles and closed triangles) indicates that if an excited state reaction occurred at high probe concentration, the product had fluorescence and motional properties in an

[2]It has been observed that phase lifetime of DPHpPC at 30 MHz is a reasonable approximation to the average lifetime based on phase and modulation measurements made at 6, 18, and 30 MHz (Parente and Lentz, 1986).

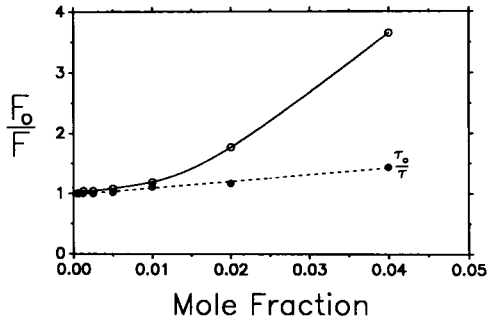

FIGURE 1 Stern-Volmer plot of fluorescence intensities (*open circles*) and lifetimes (*solid circles*). Vesicles containing different mole fractions of DPHpPC (0.25 mM DPPC) were suspended in 50 mM KCl. DPHpPC fluorescence was excited at 366 nm and the whole emission was recorded using a KV418 cut-off filter (Schott Glass Technologies Inc., Duryea, PA). Lifetimes were obtained as the average of phase shift and modulation ratio lifetimes for the sample at 30 MHz using an isochronal standard of DPH in heptane (τ = 6.77 ns; 2×10^{-7} M) as a reference for lifetime measurement (see text).

ordered lipid bilayer (DPPC at 25°C) similar to those of DPHpPC.

Excitation spectra were also recorded at numerous lipid-to-probe molar ratios. These are normalized to the 365-nm peak and plotted in Fig. 3. Unlike our results for the emission spectra, the excitation spectra did change with probe concentration in the membrane. As the concentration of the probe in the membrane decreased, so did the intensity of the 383-nm peak relative to the peak at

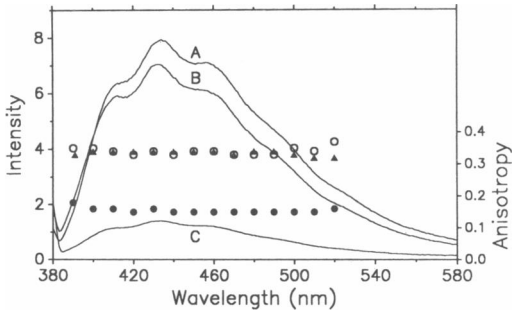

FIGURE 2 Fluorescence emission spectra of DPHpPC in DPPC LMV suspended in 50 mM KCl at 45°C. Spectra are presented for samples containing DPHpPC at lipid/probe molar ratios of (*A*) 25:1, (*B*) 150:1, and (*C*) 500:1 (0.25 mM DPPC). Fluorescence intensity is in arbitrary units. The emission spectra were corrected for color effects of the photomultiplier tube. Steady-state fluorescence anisotropy measurements were also recorded at 10-nm intervals throughout the emission band at lipid/probe molar ratios of 25:1 (*open circles*) and 400:1 (Parente and Lentz, 1985; *solid triangles*) at 25°C, and 25:1 (*solid circles*) at 45°C. A UG1 UV transmitting black glass filter (cutoff at 430 nm, 65% transmittance at 366 nm, Schott Glass Technologies Inc.) was used in the excitation path for polarization measurements. Other details in text.

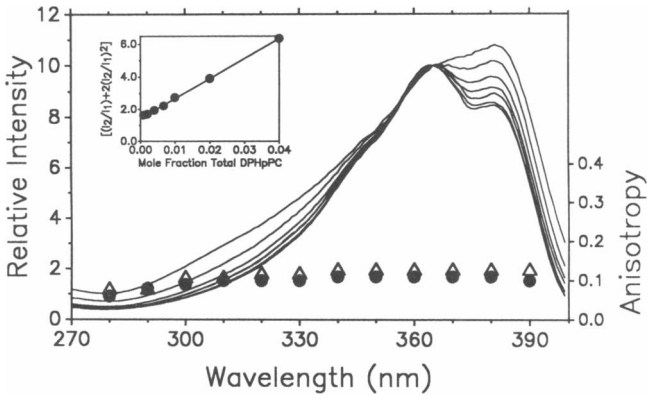

FIGURE 3 Fluorescence excitation spectra of DPHpPC in DPPC LMV at 45°C in 50 mM KCl. Spectra are presented for samples at lipid/probe molar ratios of 25:1, 50:1, 100:1, 150:1, 250:1, 500:1, and 1,000:1 (in order from highest to lowest intensity at 383 nm; 0.25 mM DPPC). The excitation spectra were recorded in ratio mode using a rhodamine quantum counter so as to correct for lamp color. Fluorescence intensity is in arbitrary units. Excitation polarization spectra were also recorded (using an SLM 4800 in the T-configuration) at 10-nm intervals across the excitation band for lipid/probe molar ratios (1.0 mM DPPC) of 25:1 (*solid circles*) and 500:1 (*open triangles*) at 45°C. Relative intensities of 365- and 383-nm peaks derived from curve fitting (described in Results) were used to determine the equilibrium constant for ground-state dimerization (*insert*). In all instances, fluorescence emission was detected using a KV418 cut-off filter (Schott Glass Technologies Inc., Duryea, PA) in the emission path to eliminate emission above ~430 nm.

365 nm. This observation suggested formation of a ground-state complex, consistent with the concave-up Stern-Volmer plot in Fig. 1. These observations led to the hypothesis that DPHpPC (and DPH for that matter) dimerize at high concentrations in a membrane (see Fig. 4). If so, the 383-nm peak should reflect, at least in part, the absorption due to a ground state dimer. We also observed that the steady-state anisotropy at 45°C across the excitation band was approximately the same at high and low probe concentrations (Fig. 3). Although this was surprising, it is not inconsistent with the proposal of dimer formation as long as the fluorescence excited-state lifetime of the dimer is several times longer than the wobbling diffusion time of this species, as is observed for the monomer species of DPH (Chen et al., 1977). Under these conditions, the fluorescence anisotropy of the probe will reflect mainly the extent rather than the rate of probe motion (Jähnig, 1979), and the extent of probe motion will reflect the molecular order within the bilayer rather than the state of aggregation of the probe.

Based on the data presented thus far, we suggest that dimer formation can account for the photophysical behavior of DPHpPC (Fig. 4). We suggest that π–π interactions within the dimer could account for the ground-state quenching seen in Fig. 1. It is necessary to assume further

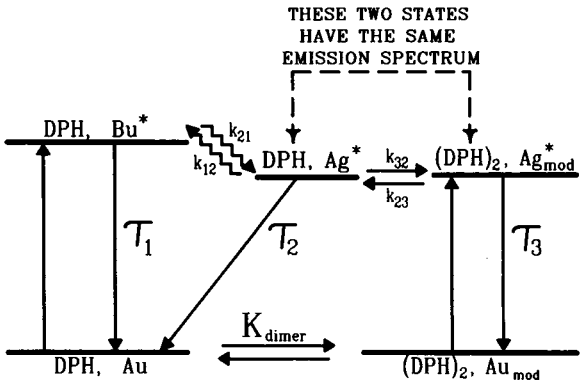

FIGURE 4 Model to explain the anomalous lifetime and photophysical properties of DPHpPC in a membrane. τ_1 represents a rapid natural decay (~1 ns) from the normally occupied $^1B_u^*$ state. τ_2 represents the much slower (~8–12 ns) decay commonly observed for DPH in membranes. This decay is slow because the $^1A_u \leftrightarrow {}^1A_g^*$ transition is formally forbidden by symmetry considerations. τ_3 represents a more rapid (~2–5 ns) decay from an $^1A_g^*$ state whose symmetry properties are presumed to be altered due to dimer formation.

that the monomer and dimer excited states had the same emission spectra, because we observed no shift in the emission spectrum of samples at high and low probe concentrations. This model is consistent with all the steady-state data presented thus far; we demonstrate below that it can account also for the complex behavior of the excited-state lifetime of DPH or DPHpPC.

Equilibrium constant for ground state dimerization

Before addressing the excited-state dynamics of our model, we must establish the equilibrium distribution of ground-state species. If our interpretation of the concentration dependence of the excitation spectra is correct, we should be able to estimate the equilibrium constant for ground-state dimerization from the intensities of the 365- and 383-nm peaks (I_m and I_d, respectively) according to the equation,

$$K = \frac{[I_d/(I_m F_r)] + 2[I_d/(I_m F_r)]^2}{X_T}, \quad (1)$$

where I_d/I_m is the ratio of 383 to 365 peak intensities, X_T is the total mole fraction of DPHpPC, and F_r is the ratio of the fluorescence efficiency (extinction coefficient*lifetime) of the monomer to the dimer. For the sake of simplicity, we assumed that the fluorescence efficiencies of the monomer and dimer were equivalent. We note that it is quite possible that the oscillator strength of the dimer would be less than that of the monomer, resulting in our underestimating the equilibrium constant for dimerization.

We resolved the intensities for the monomer and dimer peaks by assuming that each excitation spectrum was the sum of three Gaussian curves centered at 345, 365, and 385 nm. These values were determined by a second derivative analysis of the individual spectra. The spectra were fit to this curve using the "Simplex" nonlinear curve fitting algorithm (Caceci and Cacheris, 1984) to obtain height and width parameters for each Gaussian curve. We note that the 385-nm peak does not completely disappear even for a sample dissolved in an organic solvent or for very low probe concentrations (0.00125 mol fraction) in a membrane. For this reason, the relative intensity of the 385-nm peak was corrected for the intensity from a very dilute sample (0.00125 mol fraction) of DPHpPA, which should be present mainly as monomer (see Results for DPHpPA), to obtain a value roughly proportional to dimer concentration. The height of the 365-nm peak was taken as the monomer intensity and the ratio of the intensities was plotted as a function of the total mole fraction of DPHpPC according to Eq. 2 (Fig. 3, *inset*), this yielded an equilibrium constant of 60 (mole fraction)$^{-1}$. This must be considered to be only a very rough approximation to the equilibrium constant due to the assumptions we made in our analysis of the excitation spectra.

To make an estimate of how badly we might be off in our approximation, we used an estimate of the dimer extinction coefficient (obtained from the corrected 385-nm peak absorbance and the mole fraction of dimer) and an estimate of dimer lifetime from the global analysis of lifetime data (see below) to estimate the ratio of fluorescence efficiencies (F_r) needed for Eq. 1. This was used to obtain a new estimate of the equilibrium constant, which was used to obtain a new estimate of the dimer extinction coefficient and lifetime (via the global analysis using the new K). This process was repeated until it converged (three passes) on self-consistent values for the equilibrium constant (270 mol frac^{-1}) and dimer extinction coefficient (23,000 OD molar^{-1}). The monomer extinction coefficient at 365 nm was 55,000 OD molar^{-1}. Although the dimerization constant increased by a factor of 4 during this iterative process, the basic features of the global fits obtained to our lifetime data did not change. This demonstrates that the ability of our dimerization model to account for the fluorescence decay characteristics of DPH is insensitive to the assumptions made in estimating the dimerization constant.

Lifetime analysis

At least qualitatively, the altered ground and excited state symmetry properties of the dimer can account for

the observed decrease in lifetime with increased probe concentration in a membrane (Fig. 1). Thus, perturbation of the normal DPH symmetry should increase the probability of the normally symmetry-disallowed $^1A_g^* \rightarrow {}^1B_u^*$ transition (see Discussion) and thereby decrease the observed fluorescence decay time. If this line of argument is correct, the decay of DPHpPC fluorescence intensity should become increasingly nonexponential at high probe concentrations. To test for this, phase and modulation lifetime data were recorded for several molar ratios of DPHpPC to DPPC, using the SLM 48000 multifrequency fluorometer with excitation at 366 nm. The analysis of these data in terms of multiple lifetime components (Gratton et al., 1984) is presented in Table 1. A Student's *T*-test was applied to demonstrate that for high concentrations of probe in the membrane (10:1 and 25:1), a two-component description was required to adequately fit the data, while, for lipid-to-probe molar ratios of 50:1 and greater, a one-component description was sufficient (see Table 1). At high concentrations of probe, the second component (~1 ns lifetime) had a significant contribution (e.g., at 10:1 it constituted 62% of the fractional intensity). The existence of a second lifetime component seemed at least qualitatively consistent with our dimerization model. However, independent of the amount of second component, the other lifetime component continued to decrease at increasing probe concentrations (see Table 1). This indicated that it would be naive to assign the low lifetime component to dimer and that a complete "global" analysis of our phase/modulation data at different probe concentrations would be needed to establish the ability of our dimerization model to account qualitatively for the excited-state dynamics of DPHpPC.

Global analysis of lifetime data

Available global analysis software (Beechem and Gratton, 1988) allows the user to define a photophysical model, then, using a nonlinear least-squares algorithm, to determine whether or not the model can adequately describe observed data. The data in our case were the multifrequency phase-shift and demodulation ratio data acquired at 366 nm for the lifetime analysis. We fit the data to the three-species model shown in Fig. 4.

To limit the number of parameters to be adjusted during the global fitting procedure to five, several parameters were fixed at "reasonable" or experimentally accessible values. The first lifetime component was fixed at 1 ns because this is roughly the expected natural lifetime of DPH (Birks and Dyson, 1963; Birks et al., 1978). The remaining two lifetimes were expected to be ~10 ns for the monomer (τ_2) and ~2–3 ns for the dimer (τ_3), but these were allowed to adjust to obtain a best fit. The lifetime of the dimer species, which according to our

TABLE 1 Analysis of fluorescence lifetime components for DPHpPC in DPPC multilamellar vesicles excited at 366 nm

Sample DPPC/DPHpPC ratio	Temperature	No. components in analysis	Component				
			τ_1	f_1	τ_2	f_2	X^2
	°C						
10/1	45	1	1.8 ± 0.047	1.0	—	—	209
	45	2	1.3 ± 0.138	0.62	3.8 ± .723	0.38	40
	25	1	1.6 ± 0.085	1.0	—	—	650
	25	2	0.8 ± 0.102	0.44	3.5 ± .327	0.56	40
25/1	45	1	4.0 ± 0.115	1.0	—	—	188
	45	2	4.8 ± 0.200	0.92	0.98 ± .374	0.08	37
	25	1	3.4 ± 0.136	1.0	—	—	350
	25	2	4.8 ± 0.183	0.82	1.1 ± .177	0.18	14
50/1	45	1	5.5 ± 0.149	1.0	—	—	140
	45	2	3.9 ± 6.62	0.24	6.3 ± 4.26	0.76	126
	25	1	5.0 ± 0.125	1.0	—	—	120
	25	2	5.5 ± 0.113	0.97	0.29 ± .543	0.03	15
150/1	45	1	6.6 ± 0.136	1.0	—	—	52
	45	2	6.9 ± 0.118	0.98	−0.002 ± 1.4	0.02	14
	25	1	6.7 ± 0.040	1.0	—	—	5
	25	2	6.7 ± 0.055	0.999	−0.5 ± 5.6	0.001	5
500/1	45	1	7.1 ± 0.074	1.0	—	—	15
	45	2	7.1 ± 0.208	1.0	0.86 ± 12	0.00	16
	25	1	7.3 ± 0.054	1.0	—	—	8
	25	2	7.2 ± 0.073	1.0	−0.500 ± 62	0.00	9

estimated equilibrium constant should predominate at high probe concentration, was allowed to experience Stern-Volmer collisional quenching by means of an adjustable Stern-Volmer quenching constant, k_{sv}. This assumption was necessary to obtain a good fit at higher probe concentrations. The rate constant of internal conversion from the high energy monomer state ($^1B_u^*$) to the low energy monomer state ($^1A_g^*$), k_{21}, was fixed at a reasonable value of 1 ps^{-1}. The rate constant for the opposite internal conversion, k_{12}, was initially allowed to vary, but it was soon found that both k_{12} and k_{21} had little influence on the global fit as long as their ratio was maintained at ~15–20, consistent with the estimated energy difference between these states (Alford and Palmer, 1982; Itoh and Kohler, 1987). For this reason, k_{12} was fixed at $1/18^{th}$ of k_{21}. The excited-state dimerization rate constant (k_{32}) was taken to be adjustable and concentration dependent. The excited state dimer dissociation rate constant (k_{23}) was adjustable. The ground-state dimerization and dissociation rate constants were not adjusted, rather the amounts of monomer and dimer initially in the ground state were fixed for the global analysis using the equilibrium constant derived from our analysis of excitation spectra (Fig. 3 and Eq. 1).

By adjusting the five unknown parameters described above, the global analysis was performed so as to return individual squared residual values for each probe concentration as well as a global squared residual for the model. A confidence test was performed on the returned values of the parameters to determine the upper and lower limits of the parameters which are within one standard deviation of the best squared residual. These tests revealed that the squared residual surface surrounding the best fit was slowly varying, therefore a wide range of parameter values gave similar squared residuals. To obtain more information about the reason for poor parameter definition, we fixed each parameter individually over a range of values and allowed the other parameters to vary so as to determine a best fit. In this way, we determined that the τ_3 and k_{sv} parameters were closely linked, as might be expected. A "best fit" was obtained for τ_3 between 1 and 5 ns and k_{sv} ~20 $(\text{mol frac})^{-1}$. However, statistically indistinguishable fits could be obtained for τ_3 having physically unreasonable values as large as 30 ns if k_{sv} were allowed to increase dramatically and k_{23} and k_{32} were adjusted slightly so as to make dimers somewhat less favorable. All of this had the effect of producing a smaller population of low-lifetime dimer excited states, a situation not physically very different from that obtained with more reasonable values of τ_3. For this reason, we have indicated in Table 2 *A* both the parameter values resulting in a "best fit" (minimized χ^2) but also what we found to be "physically reasonable" and "stable" ranges of all parameter values, rather than statistically defined ranges.

TABLE 2 Recovered parameters from global analysis of phase/modulation data in terms of proposed model

A. Parameters for DPHpPC			
Final global chi-square*	10.43		
Local chi-square*			
10:1	20.15		
25:1	11.72		
50:1	13.78		
150:1	8.00		
500:1	8.59		
		Low‡	High‡
Lifetime 1	1.00§		
Lifetime 2	10.25	10.00	12.00
Lifetime 3	2.94	2.00	15.00
k_{sv} on lifetime 3	20.77	12.00	60.00
k_{21}	1000§		
k_{12}	55§		
k_{32} (conc. dependent)	5.37	3.40	6.90
k_{23}	1.21	0.30	2.10
B. Parameters for DPHpPA			
Final global chi-square*	2.49		
Local chi-square*			
10:1	2.14		
25:1	4.78		
50:1	1.78		
150:1	2.84		
500:1	3.56		
		Low‡	High‡
Lifetime 1	1.00§		
Lifetime 2	10.28	10.20	11.10
Lifetime 3	2.08	2.00	15.00
k_{sv} on lifetime 3	3.53	1.00	4.00
k_{21}	1000§		
k_{12}	55§		
k_{32} (conc. dependent)	0.04	0.02	0.80
k_{23}	0.19	0.10	2.00

Units: Lifetimes – nanoseconds. Rates (except k_{32}) – inverse nanoseconds. k_{32} – inverse nanoseconds inverse molar. k_{sv} – inverse units of concentration of quencher (mole fraction). *Chi-square values are reported as reduced chi-square. ‡Upper and lower limits on the parameters which still yield "physically reasonable" results (see Results). §These parameters were fixed during the analysis because the global fit was insensitive to the values.

Within these ranges, variation in the indicated parameters did not result in widely varying or physically unreasonable (i.e., negative rate or Stern-Volmer constants, $\tau > 15$ ns) optimized values of any other parameters.

The ability of our dimerization model to account for our observed phase shift and modulation ratio data is illustrated in Figure 5 for the extremes of DPHpPC concentrations considered. The fit of calculated to observed data was apparently quite good. The reduced χ^2 characterizing the "best fit" obtained for the parameters in Table 2 *A* was 10.4. The fact that this was not 1 reflects the fact that the precision of individual measurements (0.5° in phase angle, 0.005 in modulation ratio) rather than the reproducibility between modulation frequencies

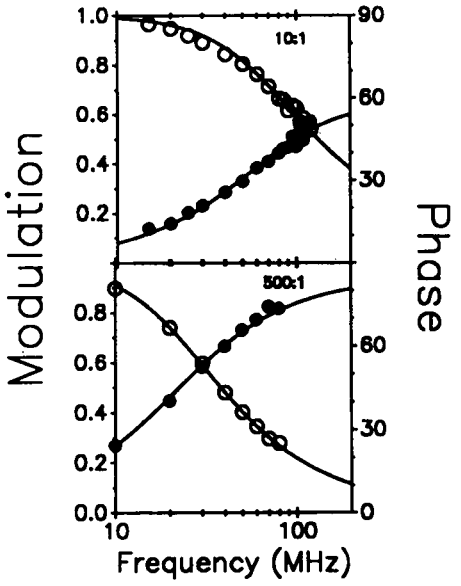

FIGURE 5 Global analysis fits of phase (*solid circles*) and modulation (*open circles*) data are shown for high (10:1) and low (500:1) probe concentration as a function of modulation frequency.

was used, as is custom, to estimate the intrinsic error in the data for the purpose of estimating χ^2. For this reason, the goodness of fit is best illustrated by Fig. 6, which summarizes the residuals of the modulation (frame A) and phase shift (frame B) data obtained for these parameters at five lipid/probe ratios as a function of modulation frequency. It can be seen that the fit to the data was quite good, with essentially a random deviation of model predictions from data over the wide range of experimental modulation frequencies and probe concentrations examined. It should be noted that the best-fit parameter values were not sensitive to the initial guesses given to the parameters; essentially the same minimum was obtained independent of the starting point in parameter space.

In general, the parameters yielding the best global fit to our data had physically reasonable values (Table 2 *A*). The lifetime values returned from the analysis agreed well with our predictions of ~10 and ~2 ns, based on the known behavior of DPH in a variety of solvents at different temperatures (Cehelnik et al., 1975) and on the lifetime observed at high concentrations in a membrane (Barrow and Lentz, 1985; Parente and Lentz, 1986). The rate constants returned for excited state dimerization yielded a dimerization equilibrium constant of 4.27 (mol fraction)$^{-1}$, as compared to the ground-state dimerization constant of 60 (mol fraction)$^{-1}$ (270 from the itterative procedure described above), making the excited-state dimer less favorable than the ground-state dimer. This is surprising because the excited state would be expected to be more polar than the ground state, which should result in stronger probe–probe interactions in the excited state. However, the packing constraints in a membrane would prevent excited DPHpPC (and perhaps DPH) molecules from having the freedom to align optimally, making the smaller excited-state dimerization constant understandable.

Photophysical behavior of DPHpPA

As a further test of our model, we compiled a similar set of observations for a negatively charged phosphatide containing DPH, namely DPHpPA. This molecule was chosen because its charge should inhibit dimerization. Therefore, our model would predict that its photophysical behavior in a membrane would be much different from that of the neutral molecule, DPHpPC. With this in mind, we obtained excitation spectra for numerous DPPC/DPHpPA molar ratios. These are plotted in Fig. 7 normalized to the 365-nm peak. Comparison of these spectra with those recorded for DPHpPC (Fig. 3) reveals a marked difference. There was not a dramatic variation between the spectra for high (25:1) and low (800:1) probe concentrations as seen in the 385-nm peak of the DPHpPC spectra. This is consistent with and supports

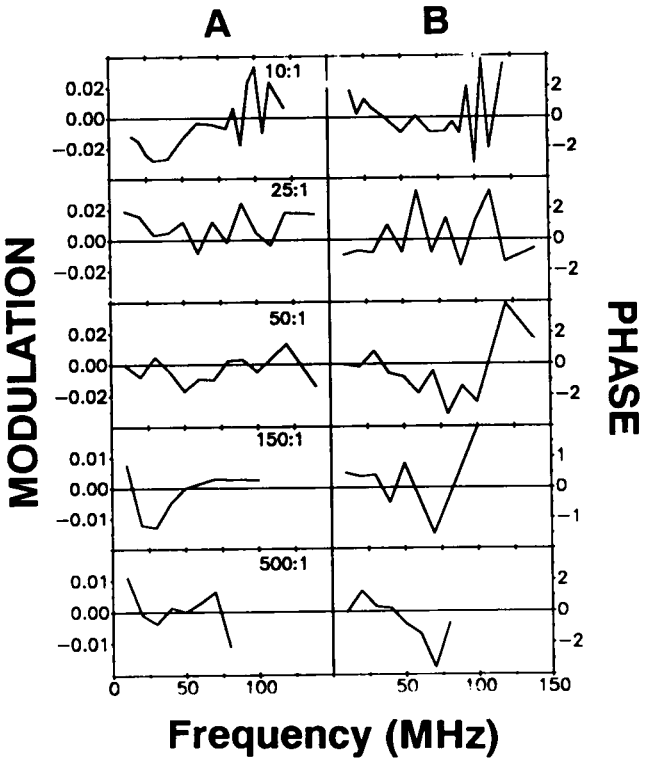

FIGURE 6 Residuals calculated from global analysis of proposed model. Residuals from all lipid/probe molar ratios are presented for modulation data (*A*) and phase data (*B*).

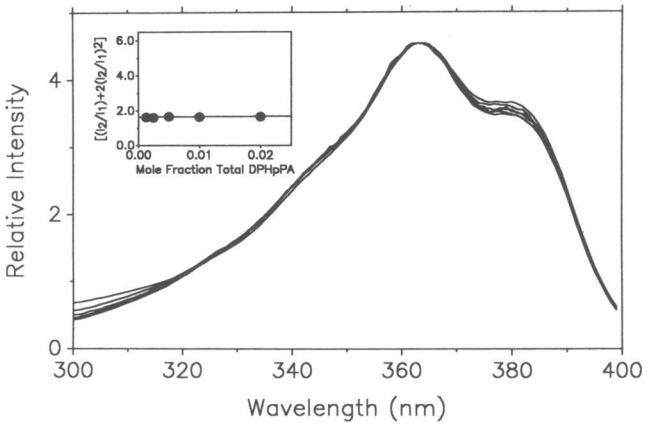

FIGURE 7 Fluorescence excitation spectra of DPHpPA in DPPC LMV at 45°C in 50 mM KCl. Spectra are presented for samples at lipid/probe molar ratios of 25:1, 50:1, 100:1, 200:1, 400:1, 800:1 (in order from highest to lowest intensity at 383 nm; 0.25 mM DPPC). The excitation spectra were recorded using the same instrumental conditions described in Fig. 3. Fluorescence intensity is in arbitrary units.

our assignment of changes in the 385-nm peak as reflecting dimer formation. The dimerization constant estimated from these data was 0.7 (mol frac)$^{-1}$ or 1.5 (mol frac)$^{-1}$ after iteration to obtain the monomer and dimer extinction coefficients (40,000 and 16,700 OD molar^{-1}; see above).

Phase shift and modulation ratio data were also recorded for several surface concentrations of DPHpPA in DPPC membranes, using the SLM 48000 multifrequency fluorometer with excitation at 366 nm. The analysis of these data in terms of multiple lifetime components (Gratton et al., 1984) is presented in Table 3. These results again show a dramatic difference in the behavior of DPHpPC and DPHpPA. DPHpPA had a much longer fluorescence lifetime at high probe concentrations than did DPHpPC, and the fractional intensity of the low-lifetime component of DPHpPA was significantly less than for DPHpPC at the same concentration. This is consistent with our original interpretation that, at high concentrations of DPH probes in a membrane, the nonexponential behavior of DPH fluorescence decay is due to dimer formation. Finally, the ground-state dimerization constant for DPHpPA was used to set the boundary conditions for global analysis of the phase shift and modulation ratio data. The results from this analysis are presented in Table 2 *B* (errors used were 0.5° in phase angle, 0.004 in modulation ratio). As for DPHpPC, the fit of calculated to observed data was good, with the major difference being in a decreased rate (k_{32}) and extent (k_{32}/k_{23}) of excited-state dimer formation. This result is not surprising given the negative charge carried by DPHpPA. The agreement of predictions of our model with physically reasonable expectations provides further support for the dimerization model proposed here.

DISCUSSION

A dimerization model is presented here (Fig. 4) to help explain the concentration dependence of DPHpPC(PA) photophysical properties. This model is based on several observations: (*a*) The concentration dependence of DPHpPC self quenching was consistent with formation of a ground-state complex along with enhanced excited state decay. (*b*) A peak on the low-energy edge of the DPHpPC and DPHpPA absorption spectra increased in intensity for increasing DPHpPC (but much less so for DPHpPA) concentration, consistent with ground-state dimer formation. (*c*) The decrease in average fluorescence lifetime with increasing DPHpPC concentration correlated with increased multiexponential character in observed DPHpPC phase shift and modulation ratio data sets. This was less evident in the case of the charged DPHpPA, which is not expected to form dimers to the same extent as

TABLE 3 Analysis of fluorescence lifetime components for DPHpPA in DPPC multilamellar vesicles excited at 366 nm

Sample DPPC/DPHpPA ratio	Temperature	No. components in analysis	Component				
			τ_1	f_1	τ_2	f_2	X^2
	°C						
10/1	45	1	5.9 ± 0.084	1.0	—	—	56
	45	2	6.8 ± 0.066	0.90	2.2 ± 0.148	0.10	0.96
25/1	45	1	6.6 ± 0.057	1.0	—	—	21
	45	2	7.3 ± 0.185	0.91	3.1 ± 0.581	0.09	2.8
50/1	45	1	6.6 ± 0.045	1.0	—	—	11
	45	2	7.3 ± 0.187	0.89	3.7 ± 0.595	0.11	1.4
150/1	45	1	6.6 ± 0.030	1.0	—	—	5.2
	45	2	7.7 ± 1.32	0.59	5.4 ± 1.13	0.41	1.9
500/1	45	1	6.6 ± 0.030	1.0	—	—	4.4
	45	2	7.9 ± 1.61	0.51	5.6 ± 1.03	0.49	1.6

DPHpPC. (*d*) Global fitting to this model of extensive phase shift and modulation ratio data sets for both DPHpPC and DPHpPA at varying surface concentrations was accomplished with physically reasonable parameter values that produced good descriptions of the data. This model is formulated so as to be consistent with the known photophysical behavior of DPH and other polyenes in organic solvents and offers an explanation for some of the anomalous properties of this fluorophore or its derivatives when they are incorporated into the hydrophobic interior of membranes.

DPH fluorescence in solvents

The characteristics of DPH fluorescence spectra have been widely studied over the past 20 or so years, both because of interest in this molecule as a membrane probe (Shinitzky and Barenholz, 1974) and because of the insight such information can offer into the photophysics of polyenes (Hudson and Kohler, 1972; Cundall et al., 1979; Cehelnik et al., 1974, 1975; Felder et al., 1982; Itoh and Kohler, 1987; Kohler and Itoh, 1988). Whereas there is wide agreement that many of the photophysical studies demonstrate an emitting excited state substantially altered relative to the initially produced excited state, many different models have been proposed to account for this. Most proposals view DPH and similar molecules as having two closely spaced excited states ($^1Ag^*$ with slightly lower energy than $^1Bu^*$). Theoretical calculations allowing for excited state mixing have shown that the normally symmetry-disallowed $^1Ag^*$ state can contribute significantly to the excited-state configuration of polyenes (Schulten and Karplus, 1972). These two very closely spaced (ΔE ~0.4–2.4 Kcal/mol; Alford and Palmer, 1982; Itoh and Kohler, 1987) and vibrationally linked (exchange time on the order of picoseconds; Felder et al., 1982) excited states would seem, at least qualitatively, to account for the steady-state spectral properties of DPH. Thus, in most commonly accepted models, excitation is via the symmetry allowed $^1A_u^* \rightarrow {}^1B_u^*$ transition, whereas emission in normal, low-dielectric solvents is dominated by the lower energy and less probable $^1A_u^* \rightarrow {}^1A_g^*$ transition (Itoh and Kohler, 1987; Kohler and Itoh, 1988).

The real-time decay of DPH fluorescence also shows anomalous behavior. All of those anomalies seem to us to be qualitatively consistent with the dual-excited state ($^1A_g^*$, $^1Bu^*$) model discussed above. The decay of DPH (or DPH-derivative) fluorescence has generally been reported to be single exponential in organic solvents (Prendergast et al., 1983; Dale et al., 1977, Cehehnik et al., 1975), although a serious effort to test a multiexponential model has been made only with paraffin oil as solvent (Dale et al., 1977). However, the long DPH fluorescence lifetime commonly observed in organic solvents, along with observed quantum yields, is consistent with an intrinsic or radiative lifetime five to six times larger (Birks et al., 1978; Birks and Dyson, 1973) than that predicted from the observed absorption spectrum using the well-established expressions of Strickler and Berg (1962). The long decay time would be consistent with emission from the slightly lower energy, but largely symmetry forbidden, $^1Ag^*$ state. Our model to account for the concentration dependence of DPH fluorescence properties accepts this basic framework and adds to it the proposal that dimerization could further complicate the observed apparent decay characteristics in a membrane environment where the fluorophore is at fairly high local concentration. Indeed, dimerization might even contribute to the distinctly nonexponential decay seen in certain solvents at very low temperature (Cehelnik et al., 1975).

DPH fluorescence in membranes

Given the anomalous decay of DPH fluorescence in organic solvents, it is not surprising that the subject of DPH fluorescence decay in membranes has been a controversial one. Whereas some studies have reported a single exponential decay of fluorescence from DPH in the fluid phase of model membrane (Shinitzky and Barenholz, 1974; Kawato et al., 1977; Klausner et al., 1980; Barrow and Lentz, 1985), others have found multiple exponential decay from such membranes, usually characterized by addition of a short lifetime component (Chen et al., 1977; Dale et al., 1977; Parasassi et al., 1984; Kinosita and Ikegami, 1984). In our view, this confusion could reflect two independent phenomena. First, dimerization could produce a lower lifetime component, as demonstrated by the work presented here. Second, we and others have reported (Parasassi et al., 1984; Barrow and Lentz, 1985) that exposure of a membrane sample to light increased the proportion of a second, short lifetime component (2 or 3 ns) associated with DPH fluorescence decay in the fluid phase. Thus, irreversible photolytic damage appears to be capable of contributing a short-lifetime component to the decay of DPH fluorescence. Others (Shinitzky and Barenholz, 1974; Duportail and Weinreb, 1983) have noted a reversible photobleaching of DPH fluorescence and proposed that this might reflect photo-induced *cis-trans* isomerization. In our efforts to extend the use of DPHpPC as a probe of membrane fusion, we have observed that irreversible photobleaching (with no change in excited-state decay characteristics) occurred more rapidly at higher probe concentrations in DPPC membranes (1.0%/min at 1 mol% probe versus 0.5%/min at 0.2 mol% probe; Burgess and Lentz, unpublished observation). This suggests that the propensity for irreversible photodamage could be related to the dimeri-

zation phenomenon described here, although much more extensive studies will be needed to test this hypothesis, including attempts to isolate and chemically characterize the photoproduct.

Another puzzling feature of the behavior of DPH in a membrane is the fact that the average fluorescence decay times of DPH or DPHpPC decrease somewhat at temperatures well below the phospholipid phase transition (Barrow and Lentz, 1985; Parente and Lentz, 1985). In the case of DPH, this drop in lifetime at low temperature could be accounted for by an increase in the proportion of the second, short-lifetime component observed below the phospholipid phase transition (Barrow and Lentz, 1985). This could reflect either decreased, thermally induced exchange from the $^1Bu^*$ to the $^1Ag^*$ state, or the occurrence of DPH or DPHpPC pools or domains in the ordered lipid matrix at low temperature (Parente and Lentz, 1985).

In summary, the model presented in Fig. 4 accounts for the concentration dependence of DPH or DPHpPC lifetime in a membrane in a manner consistent with the documented photophysical properties DPH. In addition, it provides a rational starting point for developing a quantitative description of the motional properties of these probes in a membrane. Such a description would serve as a further test of this model.

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